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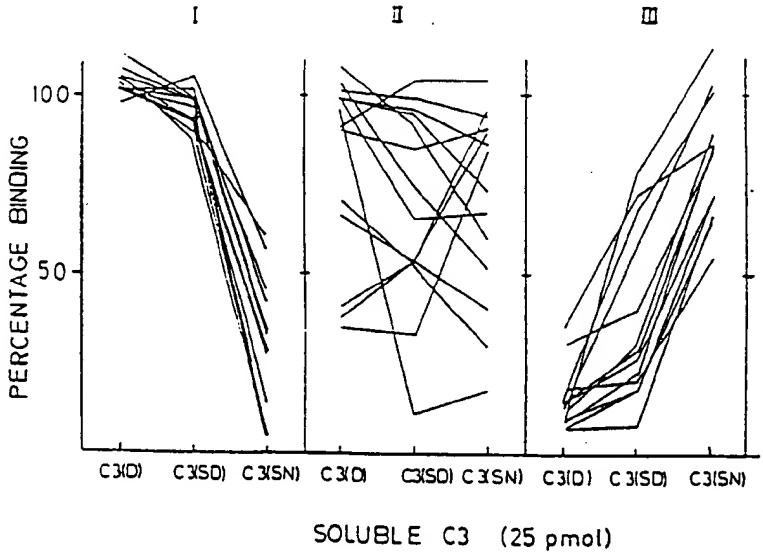
(54) Title: ANTIBODY PREPARATION DIRECTED AGAINST NEOANTIGENS IN HUMAN C3 (COMPLEMENT FACTOR 3) AND THE USE AND MANUFACTURE THEREOF



(57) Abstract

Antibody preparation directed against an individual neoantigen in the C3b region of human C3, and the use and production thereof. The preparation is characterized by immunnochemically reacting with (a) antigenic determinants in the C3b region of the alpha or beta chain of denatured human C3 in which the disulfide bonds have been reduced, but not with (b) human native C3. The use comprises immunochemically binding the preparation to C3 fragments, especially C3 fragments bound to immune complexes. The production encompasses the raising of an immune response containing the antibodies and selecting from said response the antibodies having the specificity given above.

Antibody preparation directed against neoantigens in human C3 (complement factor 3) and the use and manufacture thereof

The invention is concerned with monospecific antibody preparations directed against so-called neoantigens (= neodeterminants) in human C3 (= complement factor 3). The preparations are in the first place of the monoclonal type. Their use involves immunochemically binding anti-C3-fragment-antibody-active components thereof to the appropriate C3 fragment; this is done especially for the purpose of detecting C3 fragments, in particular C3 fragments bound to circulating immune complexes (= CIC).

The term "C3" as used hereinafter refers to human C3 unless otherwise stated. Immunoglobulin is designated "Ig".

The complement system consists of about 20 components which have to react in a well-defined reaction sequence in order that destruction of e.g. invading microorganisms can take place in the final stage. The complement system is considered to be a fundamental element of the mammal's defense against infections caused by bacteria and by viruses. The components discovered first were called "factors" and were designated chronologically C1, C2 ..... C9 all along as they were being discovered.

Complement activation can often be correlated to inflammatory processes, exposure of the factors to synthetic surfaces, surgery and diseases involving immune complex formation, e.g. infections, autoimmune diseases, cancer etc.

C3 is composed of two different polypeptide chains. They are called the alpha chain and the beta chain, respectively, and are linked together by disulfide bonds. The alpha chain is believed to have a molecular weight of 115,000 daltons and the beta chain is believed to have a molecular weight of 70,000 daltons.

context to neoantigens the generation of which is dependent on the binding of C3 fragments to various target surfaces, as for instance immune complexes. Antibody preparations directed against immunocomplexed C3 fragments have been considered to be useful for detecting C3-containing CIC and tissue-deposited C3 fragments (4, 31). Various methods for immune complex determinations have been compared (14, 15).

A number of monoclonals directed against C3 have been described. Tamerius, JD et al. (25, 26) have prepared monoclonals directed against human C3 (immunization with C3). Only one of these reacted exclusively with C3 fragments (C3d region). Lachmann, PJ et al. (10, 11, 12, 32) have described three different monoclonals. Only one of them (clone 9) reacted with neoantigen (in the C3g region). Burger, R et al (2) have produced eight different monoclonals by immunization with guinea pig C3. All the eight monoclonals reacted with native guinea pig C3 bound to plastic surfaces of microtiter wells, and two of them reacted moreover also with human C3 (clones 105 and 111). Aguado, MT et al. (1) have selected some of the aforesaid monoclonals for analyzing them in respect of their use for the detection of immune complexes and complement activation. Whitehead, AS et al. (29) have produced a monoclonal that reacts with native C3. None of the known monoclonals have been convincingly shown to discriminate between acceptor-bound fragment and the corresponding free form of the fragment.

Hansen, O. et al. (7) have described an adsorbed antiserum possessing a specificity directed against C3c epitopes. The preparation contains antibodies directed against a plurality of determinants.

The inventors themselves have shown in a number of earlier papers that determinants brought forth when C3b binds covalently to erythrocytes cell membranes can be found also in denatured C3 (16, 18, 19, 20). On the basis of these

The antibody preparations of the invention are monospecific against the individual neoantigens that are present in reduced forms of C3, in the first place in the C3b region of C3, such as the C3c or C3d region. The neoantigens in question are present either (preferably) in the alpha chain thereof or in the beta chain. An antibody preparation of the invention will thus not react with native intact C3 homologous to the immunogen used for immunization, but will react with denatured and completely reduced forms thereof, that is, with at least one of the C3 polypeptide chains dissociated from the other chain. In certain cases the preparation may also react specifically with at least one fragment (possibly bound to a target surface) selected from the group consisting of C3b, C3bi, C3c, C3d,g, C3d, C3g and other physiologically occurring C3b fragments. The preparation preferentially reacts with the fragments covalently bound to any one of the aforesaid target surfaces, but may in certain other cases also react with other forms. Particularly good are preparations with which it is possible to immunochemically assay for bound fragment in the presence of the corresponding free fragment.

The preferred antibody preparations of the invention are not significantly inhibited in their reaction with C3(D) by soluble (free) native C3b, C3bi, C3d,g or C3d fragments. For instance under the conditions given in the experimental part a more than 25 times higher, such as 50 times higher dose of the soluble fragments compared to the corresponding SDS-denatured or covalently bound fragments (molar basis) is required to effect the identical inhibition in the inhibition-ELISA.

The antibodies of the preparations may be present therein in the form of antibody-active fragments, for instance Fab, Fab', $F(ab')_2$. They may also be in the form of derivatized antibodies. The essential requirement is that the antibody fragments and the derivatives possess biospecific immune-type affinity in accordance with the present invention.

methods the specificity of the immune response can be limited. By means of a suitable immunosorbent technique it is potentially possible to obtain purified forms of the antibodies directed against the desired neoantigen. In the present stage immunosorbent purification of polyclonal antibodies to obtain the preparation of the invention is a laborious procedure which will always give low yields.

The best method for selecting the antibodies in the immune response in order to obtain a good antibody preparation according to the invention is a so-called monoclonal technique (8) by which after immunization antibody-producing plasma cells are fused with cells of a suitable myeloma cell line so that they become capable of quick and uninterrupted growth. By cloning, selecting and culturing the fused cells that produce antibodies having the specificity (C3(D)) and cross reactivity in accordance with this invention it is possible to obtain antibody preparations directed against the individual antigenic determinants which are to be found in reduced forms of C3, i.e. in the free peptide chains thereof. Cultivation of the selected cell clones for producing the antibody preparations of the invention may be carried out in cell cultures in vitro or as ascites tumors in vivo. Purification and isolation may be performed in the same manner as purification and isolation of any antibodies in general - by salt precipitation or by means of various chromatographic methods like, for instance, ion exchange, affinity, gel etc. chromatography.

Immunogens that may be employed are those that will exhibit the aforesaid particular determinants when exposed in vivo to the immune system. A surprisingly large proportion of antibodies of the specificity as prescribed according to the invention are obtained in the immune response if the immunogen is either the alpha chain or the beta chain in the denatured form, a mixture of them, or suitable immunogenic fragments

According to a second classification system, the methods may be classed as being either competitive or non-competitive methods. In a competitive method the arrangement is such that two immune reactants having a common epitope (determinant) are made to compete for an insufficient number of homologous binding sites on an immunological counterpart. In order to thus assay for a C3 fragment present in the sample and having a neoantigen against which a preparation of the invention is directed, the neoantigen of the sample is allowed to compete for the antibodies with an added reactant which carries the same neoantigen. The added neoantigen may be in a labeled, solid-phase-bound or soluble form.

Which particular form is to be chosen will be a matter subject to practical considerations, for example, with regard to the form in which the fragment is present in the sample. Competitive methods are often called "inhibition methods". In a non-competitive method arrangements are such that no competition can take place.

According to a third classification system, the methods may be classed as being either precipitation or non-precipitation methods. As examples of useful precipitating reagents may be mentioned precipitating antiserum and so-called solid-phase-bound antibodies, both of these directed against components of the complex, preferably against the antibody components. It may be mentioned in this context that neodeterminants in C3 as a rule are nonrepetitive; for this reason monoclonal antibodies directed against them are non-precipitating:

According to a fourth classification system, the methods are classed according to the marker group employed; the methods are thus radio-, enzyme-, fluorescence-, chemiluminescence-, enzyme-substrate-, immunochemical methods etc.

Among immunochemical methods may be mentioned also immuno-electrophoresis, particle agglutination, immunodiffusion and miscroscopy with labeled antibodies.

is to be measured in each individual case. If the total amount of immune complex containing a certain fragment is to be determined then the general precipitation methods for immune complexes may be employed in combination with an antibody preparation of the invention which is specific against the particular neodeterminant present in the fragment sought. If an antibody preparation is employed which reacts with all conceivable C3 fragments potentially present in immune complexes, then the result obtained will be the total content of C3 fragments bound in the immune complex. If specific components of C3 fragment-containing CIC are to be assayed for, two reagents are required differing inter se by being specific for different components of CIC. The reagent required in that case in addition to an anti-C3-fragment-antibody preparation is a reagent that is specific for the antibody portion or antigen portion in the CIC. For example, the reagent may be specific for one of the known Ig classes or for the antigen. Optionally in addition to these reagents still further reagents may be used. Assays for immune complexes containing C3 fragments have been described heretofore, (1, 21, 23, 31).

The invention is applicable to assays in various types of samples which contain C3 and/or fragments thereof. It has been known that C3 and its fragments are present in, for example, tissues and body fluids like blood, plasma, serum, urine, synovial fluid, cerebrospinal fluid etc. C3-forms having no C3(D)-determinant can in certain cases be assayed, if they are denatured before reaction with the anti-C3-preparation.

Conditions employed for carrying out the immunochemical reaction(s) are such as are usual in this type of assay methods. For instance, temperatures may be chosen within the range of 0 - 40 °C, especially 15 - 40 °C. A suitable pH is usually pH 4.5 - 10, preferably about 5 - 8.6. Quite generally, of course, measures and steps have to be taken so as to avoid activation of complement or undesired denaturation of

Mouse monoclonal antibodies

Group I: Mouse monoclonal antibodies raised against native C3 and C3b and selected for their specificity for soluble and erythrocyte-bound C3b were a kind gift from Dr Hans Müller-Eberhard, Scripps Clinic and Research Foundation, La Jolla, USA (25).

Group II: These antibodies were raised against and selected for specificity for SDS-denatured C3. Two Balb/c female mice, 8 - 12 weeks of age, were primed with 24 /ug of human SDS-denatured C3 in Freund's complete adjuvant (FCA, Behringwerke AG, W. Germany) together with 20 /ug of Lipopolysaccharide W (LPS, Difco, cat no 3120-25) subcutaneously. Eight weeks later, on day four before fusion, 100 /ug of SDS-denatured C3 in phosphate buffered saline, pH 7,4, was given intraperitoneally (i.p.). Hybridomas were produced according to standard procedures (6,9) with the following minor modifications. Four different Sp2/0 lines were used. Original Sp2/0.Ag14 (22) growing in standard Dulbecco's Modified Eagle's Medium (DMEM, Paisley, Scotland, cat no 041-1966) containing 5 % fetal calf serum (FCS, Gibco, cat no 011-6290) and 25 /ug/ml of Gentamycin (cat no G-7507, Sigma Chem Co, USA) was the progenitor line. Two different subclones selected for growth in serum-free medium and one subclone growing in low serum medium Hy-0.1 were also used. Standard DMEM containing 10 % FCS or HY-0.1 media were used for selection and cloning. The clones were screened for binding to SDS-denatured C3 in the direct-binding ELISA. Fourteen clones were randomly selected and further tested.

Group III: These antibodes were raised against and selected for specificity for denatured-reduced C3. Monoclonal antibodies in this group were produced in the same way but with the following modifications. Mice were injected s.c. with 30 /ug of denatured-reduced C3 in FCA. 30 /ug booster doses of the same antigen which was present in Freund's incomplete adjuvant (FIA, Behringwerke AG, W. Germany) were administered

One of the fourteen clones showed loss of reactivity and had stopped producing Ig. The others still had the same specificity patterns as before, with a strong specificity for C3(D) in solution which means they were directed against the neoantigens as per aforesaid definition. The presence of these neoantigens was then assayed for in physiological forms of bound C3. In order to avoid undesirable conformational changes and reduced access to the antigen surface the reactivity of the monoclonals was tested by means of inhibition ELISA and sandwich ELISA where the monoclonal antibody had been tethered to a solid phase by direct adsorption or indirectly via an anti-mouse Ig.

Preparation of particle-bound C3

$EAC14^{oxy}23b$ and $EAC14^{oxy}23bi$ cells were prepared and the uptake of C3 on the cells was estimated with ^{125}I-labelled native C3 as described before (16). 100 mg of boiled Zymosan A (Sigma Chem. Co., USA) was incubated with 2.5 ml serum containing 2 µg of native ^{125}I-labelled native C3 for 30 min at 37 °C. The uptake of C3bi was calculated from the particle-associated ^{125}I cpm.

Enzyme-linked immuno sorbent assay (ELISA)

Direct-binding assay: Serially diluted anti-C3 antibodies were allowed to bind to constant amounts of C3 or C3 fragments adsorbed to microtitre plate wells. The bound antibodies were thereafter quantified by anti-rabbit or anti-mouse immunoglobulins conjugated with horseradish peroxidase (HRP) (DAKO Immunoglobulins A/S, Denmark). Phosphate buffered saline (PBS) containing 0.1 % TWEEN 20 (v/v) and 0.1 % (w/v) bovine serum albumine (BSA) was used as a working solution.

1. 200 µl each of C3, C3 alpha and beta chains, SDS-denatured C3, C3c and C3d in PBS (corresponding to

An affinity purified Fc-specific rabbit anti-mouse Ig was adsorbed to microtiter wells (NUNC Immunoplate type I) in that 1.25 µg Ig/ml in 96 x 200 µl were incubated at +4 °C for about 16 hours or more. Then after washing the wells were incubated with a serial dilution of monoclonal (which may be unpurified supernatant), 100 µl/well, 1 hour at RT.

2. Immune complex analysis

After step 1 100 µl of a standard dilution from 1/10 to 1/10240 or plasma samples diluted 1/26 were incubated in the wells for one hour at RT. Next followed an incubation with an affinity purified Fc-specific rabbit anti-human IgG which was beta-galactosidase-conjugated and diluted 1/200 (1 hour at RT). The amount of enzyme bound to the wells was quantified with the aid of PRIST/RAST developing substance (100 µl, Pharmacia AB) which was allowed to react in the wells for two hours, whereupon the reaction was stopped after two hours with 50 µl of 0.66 M Na_2CO_3.

The wells were extensively rinsed 3 times after the incubations with the different antibodies and samples.

Immune complex model

Aggregated IgG with bound C3 was prepared in that serum is activated with an addition of 600 µg of aggregated IgG/ml and was incubated at 37 °C (14).

Samples

EDTA-plasma stored at -70 °C.

SDS-PAGE/Immunoblotting

SDS-PAGE was performed according to the method of Laemmli et al (13) and immunoblotting as described earlier (16). In

Group III was also clear-cut in that C3(D) and C3(SD) affected binding, due to the presence of C3(D) antigens. The limited inhibition caused by native C3 was probably due to a small fraction of molecules denatured during preparation and storage and the dose of antigen needed to obtain an identical degree of inhibition by C3(SN) as by C3(D) was more than 32 times higher.

Group II was more complex since the antibodies were inhibited by all forms of C3. Three of the antibodies were preferentially inhibited by C3(D) antigens while others were more affected by C3(SN) and C3(SD) antigens, suggesting that the specificity of group II antibodies was for both C3(S) and C3(D) antigens.

The specificity of the monoclonals for 2.5 pmol of soluble fluid-phase fragments expressing S and N antigens (C3b, C3bi, serum, aged serum) was also tested (figure 3). The different preparations affected the binding of each group in a similar way. Group I was considerably inhibited by all fragments while group II presented a heterogeneous pattern with a wide range of degrees of inhibition. Group III was only moderately affected by the different fragments with the exception of 4 antibodies that were inhibited to some degree by C3bi.

Specificity of monoclonal anti-C3 antibodies for particle-bound C3

The antibodies of groups I - III were tested individually by inhibition ELISA against 2.5 pmol of particle-bound C3 of $EAC14^{oxy}23b$, $EAC14^{oxy}23bi$ and ZyC3bi (figure 4). Particle-bound C3 effected inhibition (<90 % binding) of all antibodies of group I. The mean level of binding was lowest when $EAC14^{oxy}23bi$ was used. Eight to nine antibodies from group II were affected by particle-bound C3 and the mean values were identical. Eight antibodies of group III bound to bound C3. Of those only one reacted with both particle-bound C3b and

Specificity of monoclonal anti-C3 antibodies for reduced C3 fragments in immunoblotting

In immunoblotting (Table II) group I was almost completely unreactive except for one monoclonal that bound vaguely to the beta chain. Group II also bound rather poorly since only 7 out of 14 were found to be positive. With one exception these antibodies bound to the 36 kd fragment of elastase-generated C3c. All antibodies of 13 from group III bound with high affinity to alpha chain fragments. As many as 7 monoclonals bound to the 25 kd fragment and 4 to the 36 kd fragment of elastase-generated C3c and one to elastase-generated C3d(19). One antibody was positive for the 40 kd alpha chain fragment created upon SDS-denaturation at 100 °C (28) but not for reduced or unreduced C3c or C3d in immuno-blotting, suggesting that it might bind to the C3g fragment (11).

TABLE II

Specificity of groups I - III for reduced fragments in immunoblotting

	C3		C3bi	C3c		C3d
	alpha	beta	70 kd	36 kd	25 kd	
I	0	1?	0	0	0	0
II	6	0	1	6	0	0
III	13	0	ND	4	7	1

TABLE III

Summarized specificity of anti-C3(D) monoclonal antibodies selected from groups II and III

Inhibition ELISA				Immunoblotting			
Particle-bound		Soluble		C3c		C3d	Group
C3b	C3bi	C3b	C3bi	25 kd	36 kd		
-	-	-	-	-	+	-	
-	-	-	-	-	+	-	II
-	-	-	-	-	+	-	
-	-	-	-	-	+	-	
-	-	-	-	-	+	-	
+	+	-	-	-	+	-	
-	+	-	-	+	-	-	
-	+	-	-	+	-	-	
-	+	-	-	+	-	-	III
-	+	-	-	+	-	-	
-	+	-	-	+	-	-	
-	+	-	-	+	-	-	
-	+	-	-	+	-	-	
-	-	-	-	+	-	-	
-	-	-	-	-	-	+	
-	+	-	-	-[1)]	-	-	

1) Binding to the 40 kd fragment created upon SDS-denaturation at 100 °C.

Immune complex analysis in plasma samples

Several human plasmas were analyzed for the sake of obtaining a preliminary rough idea as to which levels of the immune complexes are still detectable, as to how they are distributed

in several places. Also, the range of variation is greater among the patients, which might possibly be interpreted as showing that some of the values are abnormal.

The fact that there is a group of patients lying on a lower level than normal individuals is interesting in itself and indicative of a high sensitivity of the system. Earlier anti-C3-based analyses (1, 30) and also several other immune complex analyses (14, 15) have the bulk of their normal values and many of their patients' values lying on the lowest detectable level. In particular, the lower values in patients than in normal individuals are an unusual phenomenon. If, departing from the one-dimensional view, one proceeds to plotting values from analyses with two different monoclonals into a two-dimensional system of coordinates then one will find that the differences between normal values and patients' values are amplified (Fig. 6). The variation in reactivity with respect to a plurality of epitopes is supported by the theory that the "immune complex" is a broad, summary concept encompassing a sizable amount of heterogeneity (15). The pluridimensional presentation makes it possible to distinguish for instance (a) a high quota of immunocomplexed C3d in plasmas containing low immune complex levels and therefore having a relative accumulation of metabolites deriving from a later stage of the elimination process, from (b) a plasma in which the C3d quota is more normal.

From the analyses of the fourteen C3(D) monoclonals of group III it may be assumed that also the rest of the expanded monoclonals will react preferentially with epitopes on C3(D) in solution, and that they will react to a large extent with neoantigens occurring on physiological forms of particle-bound C3. A significant portion of these neoantigens are probably to be found on C3 associated with immune complexes or models thereof in the form of aggregated human IgG. Moreover, they vary inter se in their specificities for various types of immunocomplexed C3. As shown by preliminary tests they exist in at least two variant forms. For this

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Figure 1: Serial dilutions of C3(SN)(O), C3(SD)(o) and C3(D) (Δ) were allowed to compete for binding to rabbit polyclonal anti-C3(D)(I) and antisera raised in mice against SDS-denatured (II) and denatured-reduced C3(III) in the inhibition ELISA.

Figure 2: 25 pmol of C3(SN), C3(SD) or C3(D) was allowed to compete for binding to the individual monoclonal antibodies of groups I - III in the inhibition ELISA.

Figure 3: 2.5 pmol of C3b, C3bi, C3 of normal human serum (NHS) and C3 fragments of aged human serum (AHS) was allowed to compete for binding to the individual monoclonal antibodies of groups I - III in the inhibition ELISA. The mean values ± SEM for the different groups in response to each C3 preparation were presented.

Figure 4: 2.5 pmol of particle-bound C3 of $EAC14^{oxy}23b$, $EAC14^{oxy}23bi$ and ZyC3bi was allowed to bind to the individual monoclonal antibodies of groups I - III in the inhibition ELISA.

Figure 5: Histogram showing percent distribution of patients material (a) and normal material (b) when analyses are carried out using the three monoclonal examples mentioned in the text. The antibody in analysis I is directed against a neoantigen exposed on bound C3b and C3bi and located in the C3c portion. In analysis II, the antibody is directed against a neoantigen exposed on bound C3bi, located in C3d,g, and in analysis III the antibody is directed against a neoantigen exposed on bound C3bi located in the C3c portion.

Figure 6: Analysis responses from the same analyses as in Fig. 5 plotted against each other. Normal material is represented by filled-in rectangles, and patients material is represented by empty circles.

Claims

1. Antibody preparation directed against an individual neoantigen in the C3b region of human C3, characterized by immunochemically reacting with

   (a) antigenic determinants in the C3b region of the alpha or beta chain of denatured human C3 in which the disulfide bonds have been reduced,

   but not with

   (b) human native C3.

2. Antibody preparation according to claim 1, characterized by immunochemically reacting with at least one region which is comprised within the C3b regions of said chains and which is selected from the group consisting of the C3bi, C3d,g, C3d, C3g and C3c regions.

3. Antibody preparation according to claim 1 or 2, characterized in that the antigenic determinant with which it reacts immunochemically is located in a C3 region corresponding to a physiologically occurring covalently bound C3 fragment on a circulating immune complex.

4. Antibody preparation according to any of claims 1 - 3, characterized in that the antigenic determinant with which it can react immunochemically is not accessible for reaction in soluble (free) physiological C3 or C3 fragments but is accessible for reaction when these fragments are bound to circulating immune complexes.

5. Antibody preparation according to any of claims 1 - 3, characterized by having been produced in that cells potentially capable of producing antibodies - said antibodies reacting immunochemically with both (a) a

FIG 1

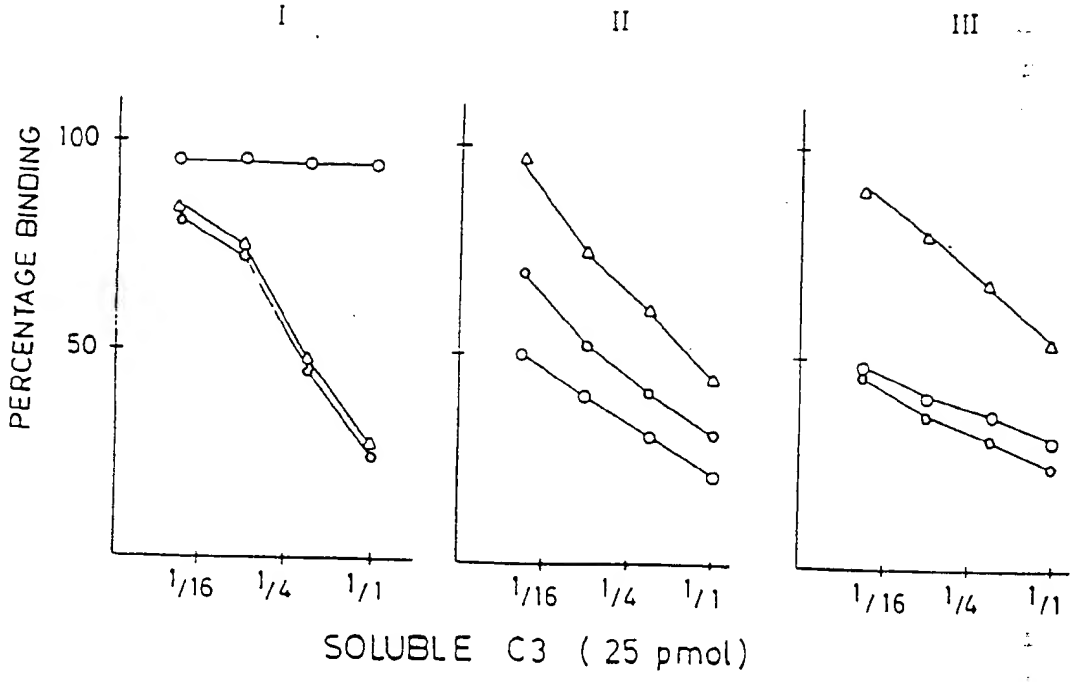

I
II
III
PERCENTAGE BINDING
100
50
1/16
1/4
1/1
SOLUBLE C3 (25 pmol)

FIG 2

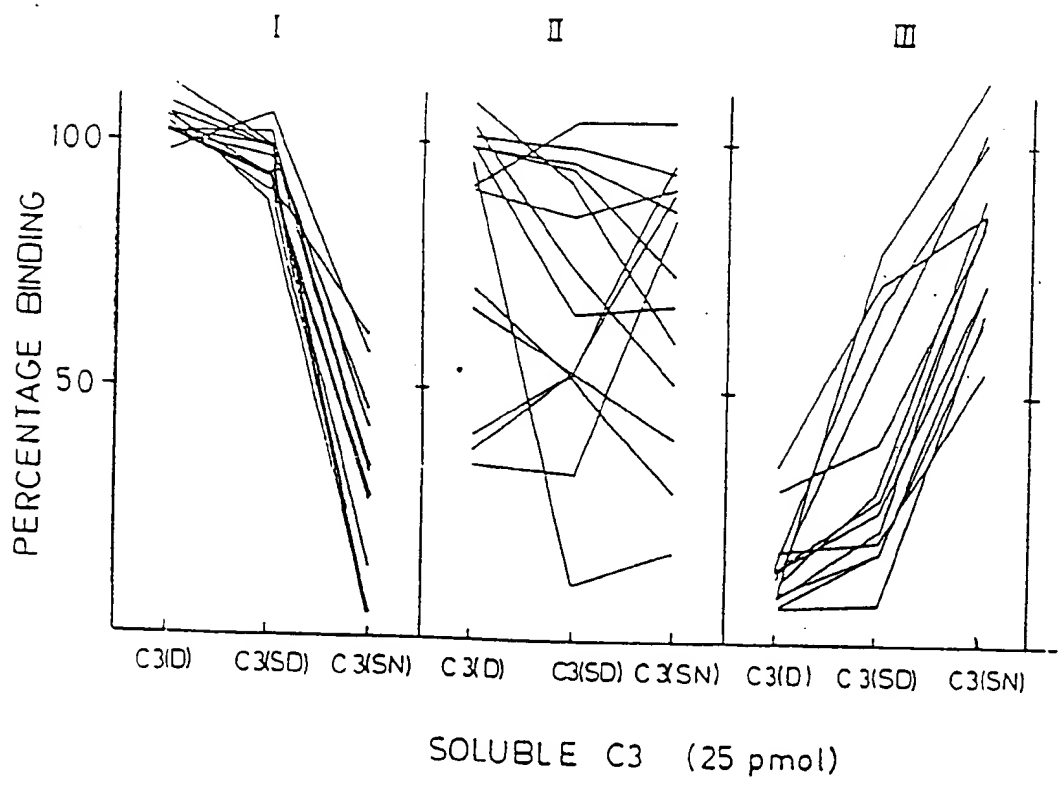

I
II
III
PERCENTAGE BINDING
100
50
C3(D)
C3(SD)
C3(SN)
SOLUBLE C3 (25 pmol)

FIG 5

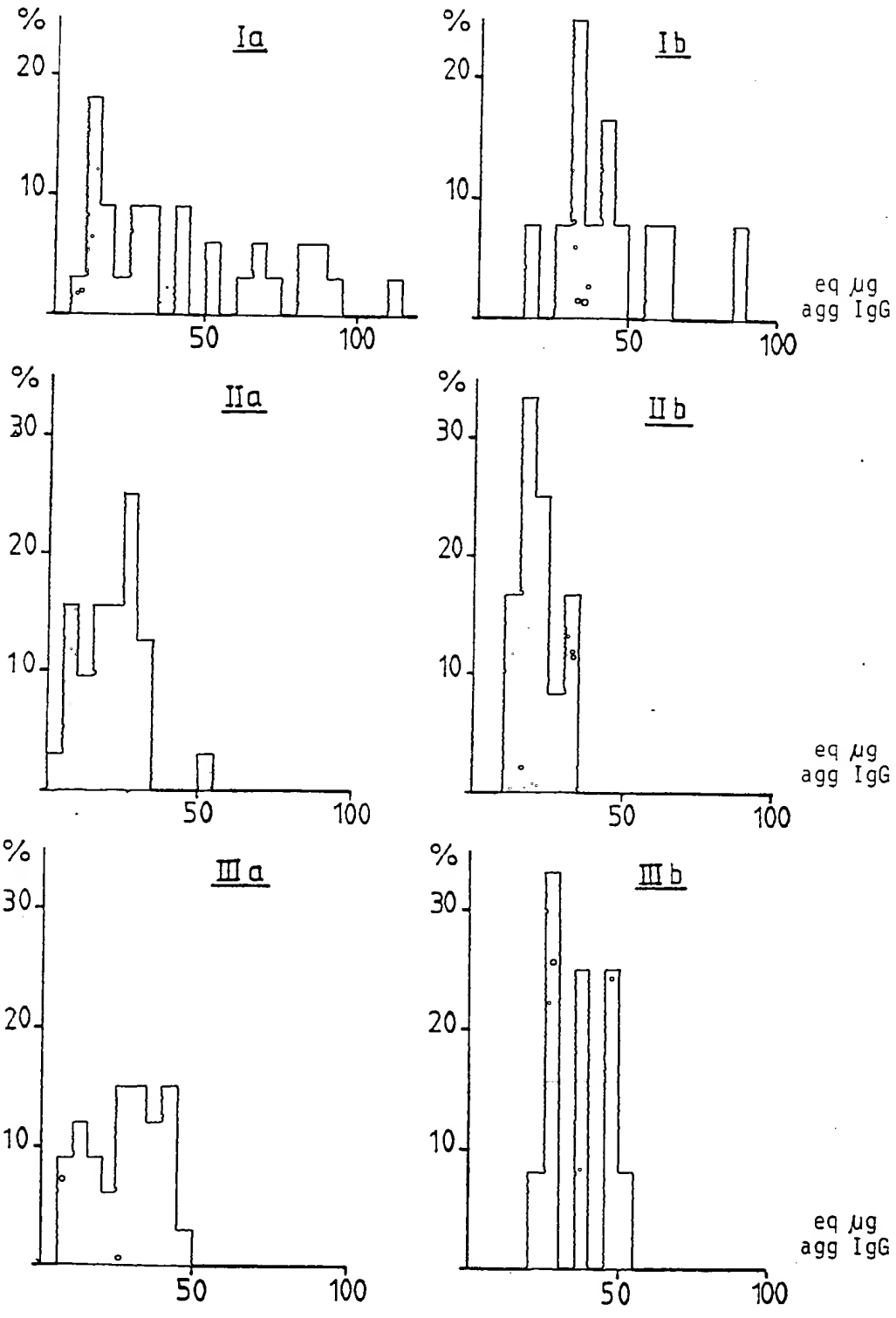
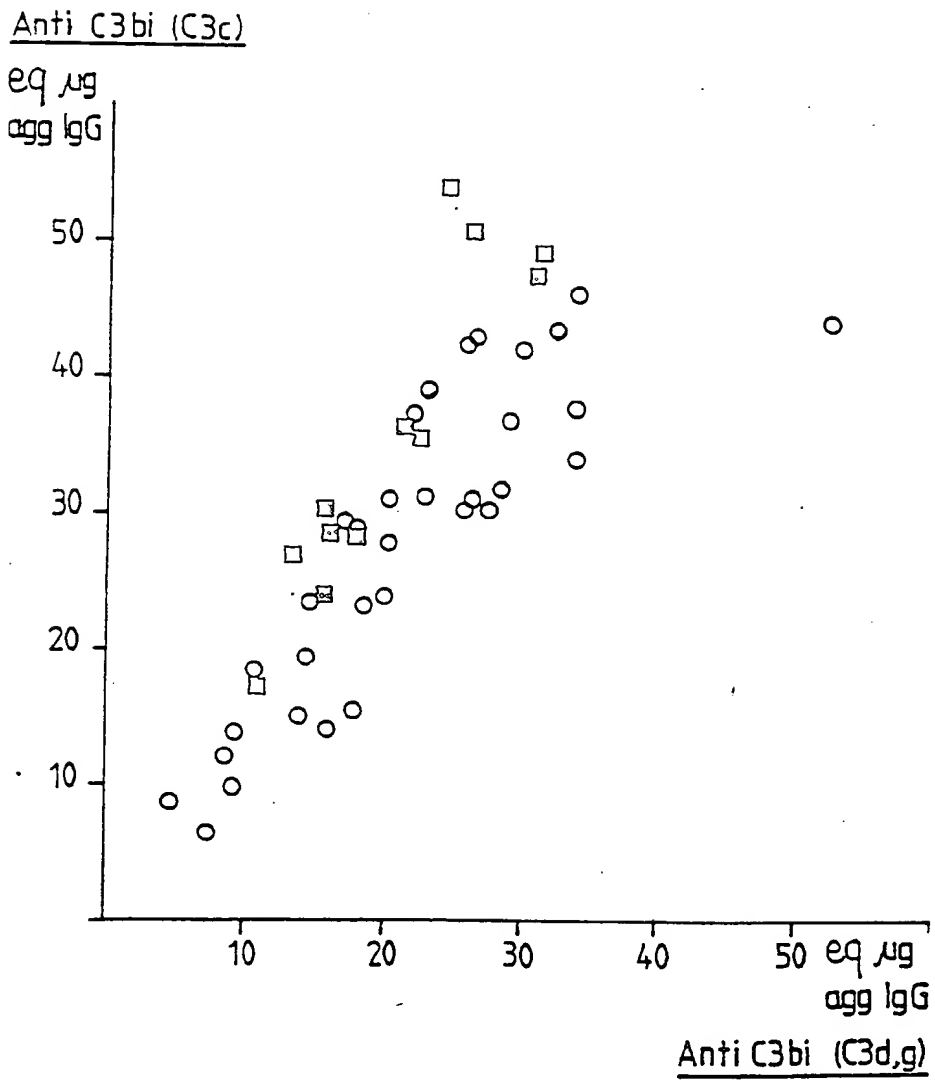


FIG 6.2


Anti C3bi (C3c)
eq µg
agg IgG
50
40
30
20
10
10
20
30
40
50 eq µg
agg IgG
Anti C3bi (C3d,g)

III. DOCUMENTS C NSIDERED TO DE RELEVANT (CONTINUED FR M THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Chemical Abstracts, vol 100 (1984), abstract No 101 357k, J Immunol 1984, 132(2), 809-15	1-7
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P	Electrophoresis 1986, 7, 379-386, Jørgen Folkersen, "Immunoblotting analysis of the Peptide Chain Structure of the Physiological Breakdown Products of the Third Component of Human Complement"	1-7

FIG 1

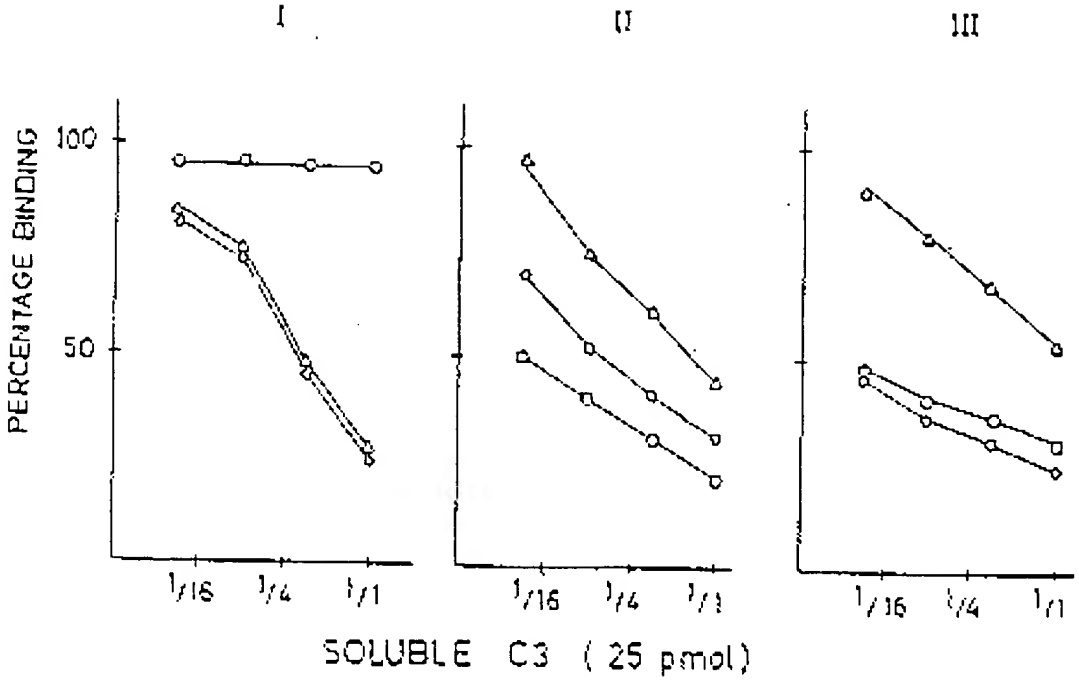


FIG 2

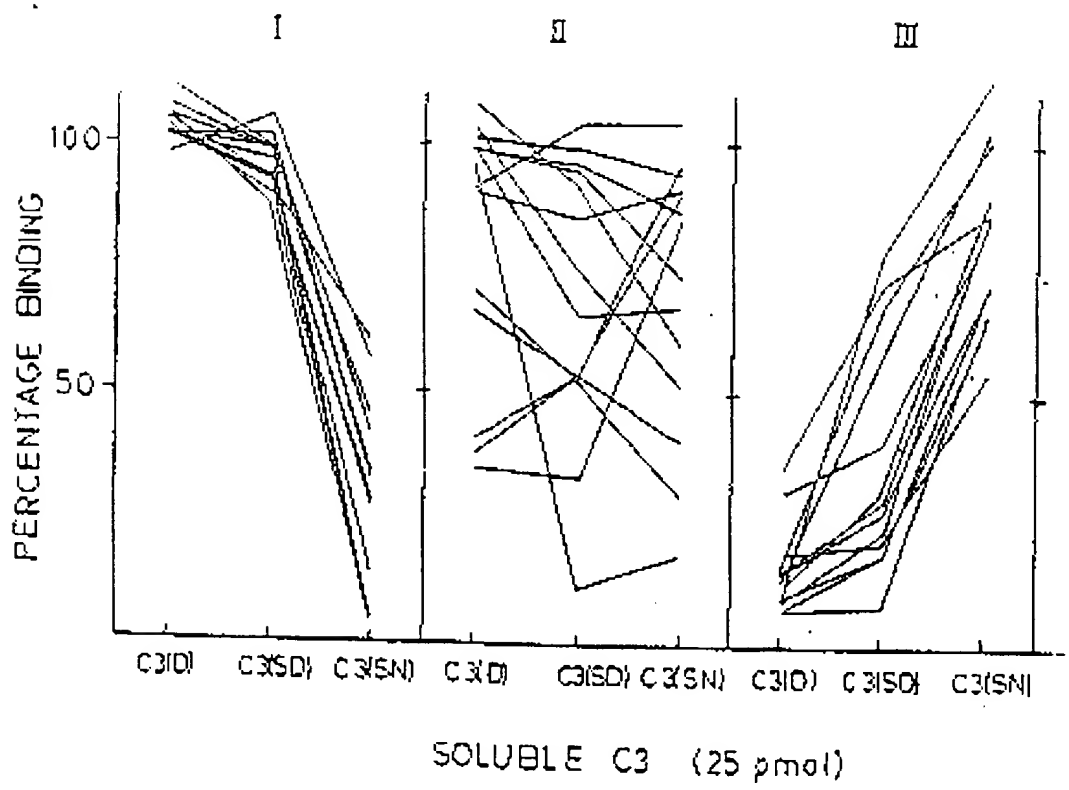


FIG 5

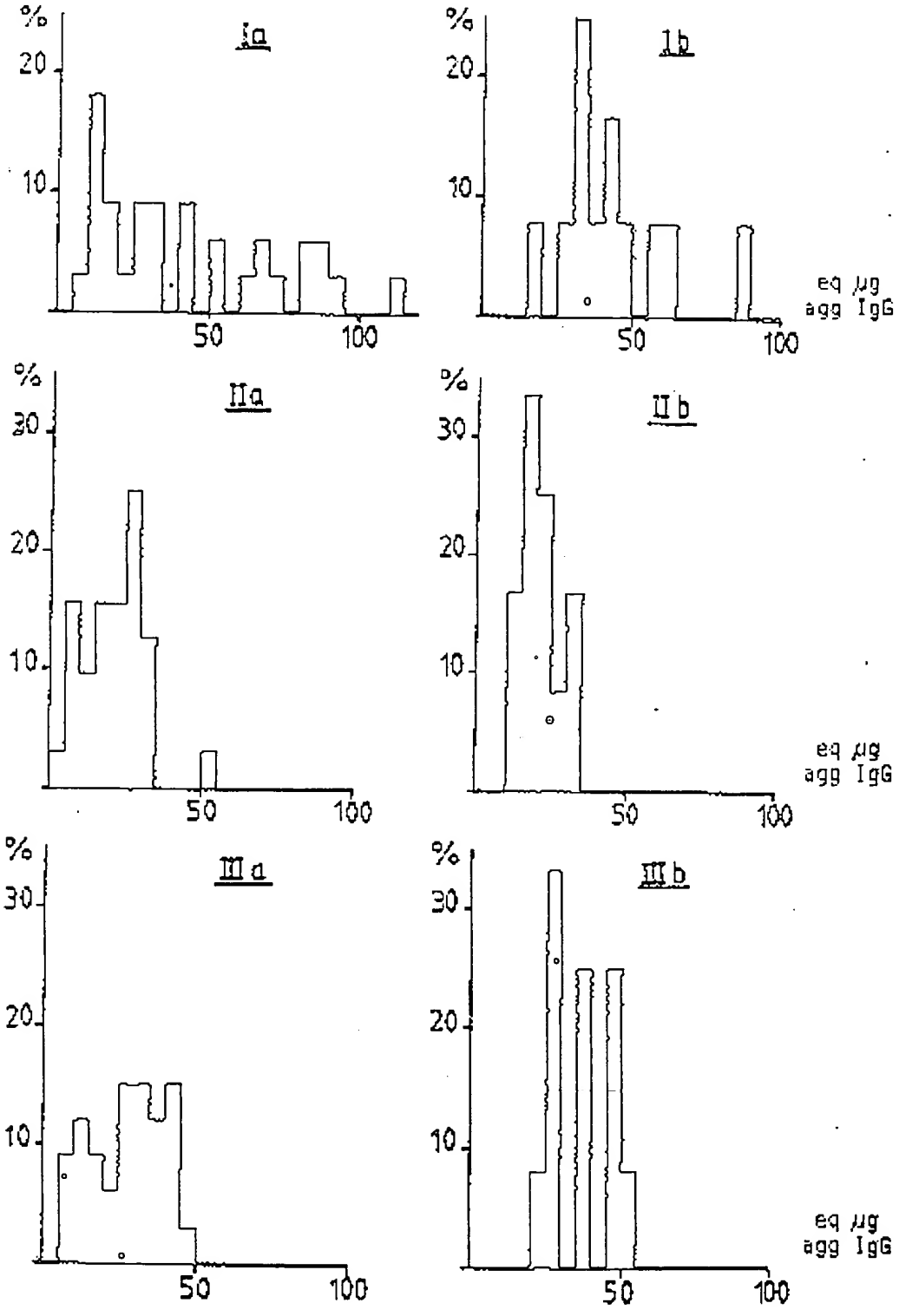
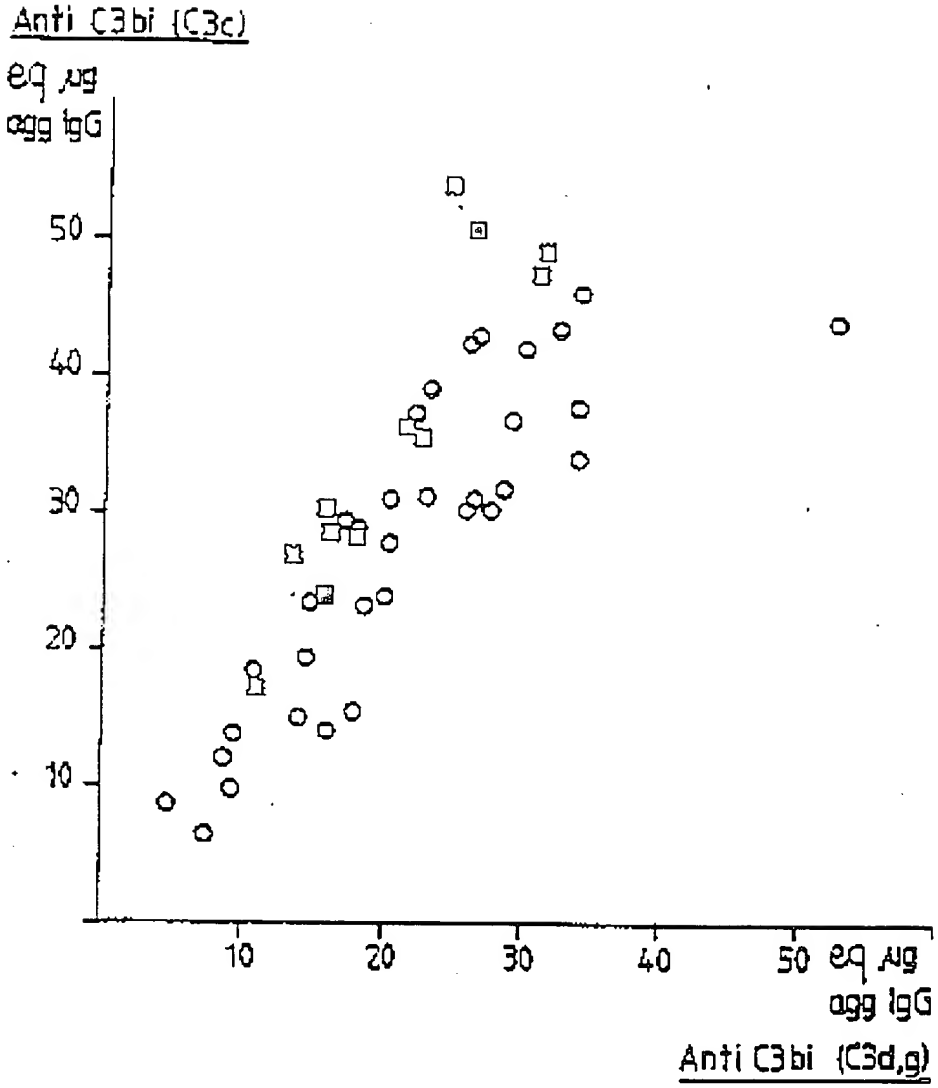


FIG 6.2


Anti C3bi (C3c)
ega ang
agg IgG
50
40
30
20
10
10
20
30
40
50 ega ang
agg IgG
Anti C3bi (C3d,g)